GENETIC ISOLATION AMONG SIX STRAINS OF *DROSOPHILA REPLETA* FROM THE EASTERN UNITED STATES, CENTRAL AMERICA, HAWAII, AND AUSTRALIA

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GENETIC ISOLATION AMONG SIX STRAINS OF DROSOPHILA REPLETA

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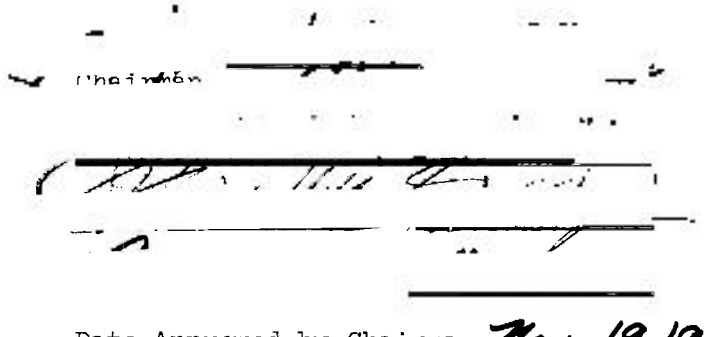
Approved:



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SUMMARY

Geographical isolation of populations reduces or eliminates the opportunity for such populations to interbreed or to share their gene pools. Thus, geographical isolation may provide the opportunity for fixation of genetic differences among populations derived from a common ancestor, i.e., for genetic isolation among such populations, which may lead ultimately to speciation. The present study of genetic isolation among six strains of Drosophila repleta from Atlanta, Georgia; Oahu, Hawaii; Yucatan Peninsula, Mexico; Palmar, Costa Rica; Sydney, Australia; and Prospect, Connecticut included determination of the extent to which the strains are genetically isolated, as indicated by frequency of successful interstrain matings relative to controls, and examination of several potentially operating mechanisms of reproductive isolation which may have arisen during fixation of genetic differences among geographically isolated populations. Strains from Georgia, Hawaii, Mexico, and Connecticut form a group with a common morphological pattern of reproductive structures and interbreed without difficulty. Strains from Costa Rica and Australia exhibit several morphological and developmental differences consistent with genetic isolation and reflecting a departure from the major type. Both strains are reproductively isolated from all other strains by sexual isolating mechanisms; thus, genetic isolation apparently has arisen in conjunction with geographical isolation among these strains.

CHAPTER I

INTRODUCTION

Investigations into the evolution of outbreeding sexual populations must concern themselves with the mechanisms by which populations adapt, diverge, and form new breeding units, which, in their turn, must adapt to the rigors of new or changing environmental conditions. This adaptation and divergence is the process of species formation, the dynamic interaction of environmental pressures and mechanisms for the initiation and preservation of induced genetic variation. There exist many different opinions as to which combination of attributes is necessary to delineate a species. In this study, when an array of interbreeding Mendelian populations becomes segregated into two or more reproductively isolated arrays, then speciation is said to have occurred (Dobzhansky, 1970). For an ancestral species to evolve into two or more derived species, some degree of reproductive isolation must exist.

Geographical isolation can be considered to be independent of the genetic constitution of the organism. A frequently encountered precursor to reproductive isolation is geographical isolation in which a physical barrier occurs in the habitat of the organism. A mountain range, an ocean, a river, or any other topographical feature which prevents contact between populations can induce isolation between them (Patterson and Stone, 1952). Another type of geographical isolation occurs when a species inhabits such a large geographical area that the

most widely separated populations do not have the opportunity to interbreed. Although there may exist a continuum of interbreeding populations intermediate to the population extremes, as this continuum canalizes into new niches, new species can evolve from races adapted to local conditions (Carson, 1959). This form of geographical isolation is commonly encountered in studies of speciation in the genus Drosophila (review by Patterson and Stone, 1952; Carson, 1959). With the exception of geographical isolation, all reproductive isolating mechanisms are to some extent dependent upon the genome of the species (Dobzhansky, 1970). These mechanisms generally are collectively designated genetic isolation.

Genetic isolation is described as comprising the following mechanisms (modified after Patterson and Stone, 1952; Eaton, 1970; Dobzhansky, 1970):

A. Pre-copulatory Isolating Mechanisms

1. Sexual isolation, or failure to mate. Sexual isolation may result from behavioral isolation, in which one member of a pair refuses to undergo (female) or initiate (male) copulation with the other member. In Drosophila, the discriminating member is usually the female.

2. Mechanical isolation, in which structural differences in genitalia preclude successful copulation.

3. Ecological isolation, in which members of different populations are prevented by ecological factors from maintaining sufficient contact to interbreed.

4. Temporal isolation, in which differences in seasonal or diurnal breeding cycles prevent interbreeding.

B. Post-copulatory Isolating Mechanisms

1. Gametic mortality, in which the alien female tract is so hostile to the male gamete that sperm mortality occurs prior to fertilization.

2. Hybrid mortality, in which death of the hybrid offspring occurs before it reaches reproductive age.

3. Hybrid infertility, in which the hybrid is incapable of producing offspring or in which the number of offspring is markedly reduced.

Of the mechanisms which maintain a degree of reproductive isolation sufficient to allow establishment of genetic differences, pre-copulatory mechanisms entail less expenditure of time and energy on the part of the species, as well as the individual, than do post-copulatory mechanisms; thus, they are biologically the more efficient. For example, hybrid infertility, a post-copulatory isolating mechanism, utilizes all the resources necessary to produce a fertile individual of either population but results in a hybrid individual which does not contribute to the gene pool of the subsequent generation. Sexual isolation, by preventing copulation between members of different species or populations, conserves time and energy which otherwise would have been expended in copulation, fertilization, and development of an infertile hybrid; thus, sexual isolation provides for more efficient utilization of resources (i.e., reproductive adults) available to produce the subsequent generation of individuals in each population.

The genetics of the genus Drosophila has been studied exten-

sively for many years. Those attributes which make Drosophila a useful experimental organism for genetic study render it a useful tool in the study of speciation. Small size, short generation time, and large numbers of offspring per female, characteristics which facilitate culture of Drosophila under laboratory conditions, also enhance success in pioneering of new habitats. The genus Drosophila has undergone extensive speciation. There are 750 known species of Drosophila (Dobzhansky, 1970), with as many as 133 species endemic to a given (Neotropical) region (Patterson and Stone, 1952). The genus has been subdivided into eight subgenera, and the larger subgenera have been divided further into species groups (Patterson and Stone, 1952; Dobzhansky, 1970).

Many investigators have studied evolutionary relationships within the genus Drosophila (reviews by Patterson and Stone, 1952; Dobzhansky, 1970). Briefly, the primitive Drosophila archetype is believed to be represented by the relict species, D. pinicola. From this ancestral type, the subgenera Sordophila, Hirtodrosophila, Drosophila, and Sophophora arose through separate lines, while Phloridosa, Pholadoris, Siphlodora, and Dorsilopha arose from lines within the subgenus Drosophila.

Within the subgenus Drosophila, the repleta species group exemplifies the extensive speciation occurring within the genus. The repleta group contains 52 species, most of which are autochthonous to specific regions and which include the majority of desert-dwelling species (Patterson, 1943). Only two species of the repleta group, D. hydei and D. repleta, are cosmopolitan (Patterson and Stone, 1952).

In the genesis of species groups of this nature, following the origin and establishment of the ancestral panmictic population and prior to its divergence into completely isolated species, the population must have undergone less sharply defined gradients of isolation. In Drosophila, some evidence of these gradients consists of the existence of chromosomal races and sibling species (Dobzhansky, 1970). Chromosomal races are populations within a species having characteristic and strain-specific frequencies of chromosomal aberrations, such as inversions, detectable in preparations of polytene salivary gland chromosomes. Several chromosomal races in D. pseudoobscura have been investigated intensively. For example, Dobzhansky (1944, 1958) analyzed several third chromosome rearrangements, including Pike's Peak and Arrowhead inversions, in populations of D. pseudoobscura in the southwestern United States and studied seasonal and long-term changes in their frequencies. The frequencies of these inversions were found to vary from population to population in a manner consistent with expectations if some degree of isolation were operating in these populations (Dobzhansky, 1958, 1970).

Sibling species are often sympatric and are morphologically very similar; nevertheless, they form distinct breeding units in nature. Examples of such species are to be found in the sibling species D. pseudoobscura and D. persimilis (Dobzhansky, 1970) and in the superspecies, or species complex, originally classified as D. paulistorum (Richmond, 1972).

Wharton (1942) constructed a detailed cytological map of the salivary gland chromosomes of D. repleta and found other species in the

repleta group to be chromosomally so similar that the D. repleta map can be extrapolated to describe the chromosomes of other species in the group by indicating areas of rearrangement of the banding patterns. The polytene chromosomes of D. repleta appear as five strands of more or less equal length (chromosomes X-5) and a dot (chromosome 6). This is considered to be the primitive chromosome number for the genus (Sturtevant, 1940, 1942; review by Patterson and Stone, 1952). In metaphase squash preparations of larval ganglia the chromosomes appear as five paired rods and a pair of dots. Metaphase chromosomes do not exhibit the morphological similarity found in salivary gland chromosomes (see Wharton, 1943 for analysis). No inversions have been detected in D. repleta (Stone, 1962).

Because of its apparently primitive position in the scheme of evolution in the genus Drosophila and because of its cosmopolitan distribution, D. repleta, together with D. hydei, is considered to be a co-founder of the large and complex repleta group (Patterson and Stone, 1952). If, as seems likely, the species of the repleta group evolved at least in part by divergence from an ancestral form identical or similar to D. repleta, it should prove instructive to examine genetic isolating mechanisms potentially operating in geographically isolated strains of D. repleta which might have resulted in the evolution of many specialized and geographically restricted species from a cosmopolitan and adaptively general form.

Wharton (1942) crossed six strains of D. repleta, including laboratory cultures collected from Fredericksburg, Elgin, and Eagle Pass, Texas; New Haven, Connecticut; Guatemala; and Ankara, Turkey.

In experiments utilizing small mass matings (5 pairs per bottle) and large mass matings (25 pairs per bottle), she observed sexual isolation among the strains studied. Upon dissection of females, it was determined that insemination had not taken place in any of the crosses yielding no offspring. Salivary gland chromosomes of F_1 larvae obtained from successful crosses and from crosses in which only one reciprocal mating was successful exhibited no chromosomal rearrangements, even in crosses between strains which were widely separated geographically. In successful crosses, the F_1 and F_2 offspring were often more fertile than either the P_1 cross or the parental controls. Wharton (1942) found no correlation between geographical origin of strains examined and the extent of isolation between them. For example, the Ankara, Turkey strain mated successfully with all other strains in both reciprocal crosses, but the Guatemala strain exhibited differential success in reciprocal crosses, dependent upon the sex of the Guatemala parent. Matings between Guatemala males and females of other strains were successful in all crosses, while Guatemala females mated successfully with males of only two of the other strains, Fredericksburg and Ankara. By contrast, matings involving females of the Elgin, Texas strain were successful in all cases, but the males crossed only to Ankara and Eagle Pass females. In no case was complete reproductive isolation between strains observed.

The present study is an investigation of genetic isolation among six strains of D. repleta, collected in geographically distant locations different from the origins of populations studied by Wharton (1942, 1943).

The strains investigated include wild type stocks from Atlanta, Georgia (ar 4), Oahu, Hawaii (ar 5), Yucatan Peninsula, Mexico (ar 6), Palmar, Costa Rica (ar 11), Sydney, Australia (ar 12), and Prospect, Connecticut (ar 13). The study includes determination of the extent to which the strains are genetically isolated, as indicated by frequency of successful interstrain matings relative to controls, and examination of several potential mechanisms of reproductive isolation which may have arisen during fixation of genetic differences among geographically isolated populations, including mechanical isolation (differences in internal and external reproductive structures among strains), sexual isolation (failure to mate, as determined by examination of females for insemination after exposure to males), hybrid mortality (as determined by reduction in frequency of successful matings and in frequency of F_1 offspring per female relative to controls), and hybrid sterility (as determined from relative success of $F_1 \times F_1$ matings as compared to controls).

In crosses in which females were inseminated but produced no adult offspring (or in which the mean number of adult offspring per female was significantly lower than in control matings), petri dish cultures have been examined for the presence of eggs, each larval instar, and pupae in an attempt to determine the stage at which development was interrupted. A potential cytogenetic basis for developmental arrest has been investigated in lacto-acetic orcein squash preparations of polytene chromosomes from salivary glands of third instar larvae of each parental strain.

CHAPTER II

MATERIALS AND METHODS

Stocks and Culture Medium

The stocks of strains used in this study were established in 1968 and 1970 from the collection of Dr. Anita I. Bolinger, Georgia State University, Atlanta, Georgia, and have been maintained in laboratory culture by Dr. Ann M. Colley at the Georgia Institute of Technology since their establishment. Origins of strains used in the study are listed below; symbolic stock designations are those of Dr. Bolinger.

(a) ar 4, Atlanta, Georgia: Derived from a stock collected by A. M. Colley at Georgia State University in 1965 and 1966.

(b) ar 5, Oahu, Hawaii: Collected in Oahu, Hawaii in 1969; obtained from Washington University, Saint Louis, Missouri in 1970.

(c) ar 6, Yucatan Peninsula, Mexico: Obtained from L. H. Throckmorton, University of Chicago in 1970.

(d) ar 11, Palmar, Costa Rica: Obtained from Genetics Foundation, University of Texas, Austin, Texas in 1970.

(e) ar 12, Sydney, Australia: Obtained from Genetics Foundation, University of Texas, Austin, Texas in 1970.

(f) ar 13, Prospect, Connecticut: Obtained from Yale University, New Haven, Connecticut in 1970.

The culture medium used for maintenance of stocks and for all experiments in the study consisted of Instant Drosophila Medium (Carolina

Biological Supply Company), supplemented with Fleischmann's active dry yeast.

Pair Matings

Pair matings were used in experiments designed to obtain the following information:

(1) Number and frequency of successful pair matings per interstrain cross.

(2) Number of offspring per pair mating for calculation of mean number of offspring per female per interstrain cross.

(3) Number of successful $F_1 \times F_1$ matings per interstrain cross. Frequencies for each interstrain cross were compared to frequencies for control crosses with respect to both male and female parents.

Parental flies were collected within 24-48 hr intervals and were aged in 4 oz bottles for 5-7 days. Females and males were aged separately. Matings were between individuals of approximately the same age (within 2-3 days). Bottles used for aging were retained after removal of flies for matings and were examined for offspring periodically for a minimum of 3 weeks to confirm virginity of females used in matings.

Pair matings were set in 18 x 150 mm Kimax culture tubes; tube cultures were incubated at 23 $\pm$ 1 ^{o}C and were examined periodically for the presence of offspring. Offspring were counted at 21, 28, and 35 days after setting of cultures. $F_1 \times F_1$ crosses were set in 4 oz French square bottles, using 4-5 females per bottle whenever possible. The number of females used in setting $F_1 \times F_1$ crosses was recorded in all experiments.

Mass Matings

Two sets of experiments involving mass matings were designed to provide the following information:

(1) Set I: Number and frequency of females inseminated per cross.

(2) Set II: Stage(s) at which development was arrested in crosses in which females were inseminated but produced no adult offspring.

(3) Sets I and II: Mean number of offspring per female per interstrain mass mating for comparison with data from pair matings.

Mass matings were set in 8 oz Boston round bottles in duplicate sets of 30 pairs per cross. Flies were aged 5 days prior to setting bottle cultures.

Set I: Mating and oviposition were allowed to continue for 7 days. Bottles were cleared on the eighth day, and females were examined for insemination. Offspring were counted, and mean number of offspring per female per cross was calculated.

Set II: Mating and oviposition were allowed to continue for 7 days. Parental flies were then transferred to egg laying chambers designed to permit oviposition on food in 150 x 15 mm petri dishes (Falcon Plastics). Offspring emerging in bottle cultures were counted, and mean number of offspring per female per cross was calculated. Petri dish cultures were examined daily to determine (a) whether oviposition had occurred and (b) the developmental stage(s) at which mortality occurred.

Detection of Developmental Arrest

Male and female parents were recovered from bottle cultures of those crosses of set II mass matings which had produced no adult offspring. These flies were etherized and transferred to oviposition chambers designed by M. K. Jocoy; the chambers were constructed from 1 qt containers of clear plastic fitted with aluminum wire mesh bases. Oviposition chambers were placed on filter paper discs in 150 x 15 mm petri dishes containing Instant Drosophila Medium to which blue food coloring had been added. The blue food coloring enhanced the visibility of eggs and first instar larvae; the filter paper discs prevented eggs from sinking into the medium.

Females were allowed to oviposit for three days; then, cages were removed and petri dishes were covered. Petri dish cultures were examined at 24 hr intervals for a minimum of 15 days, the mean duration of the life cycle calculated for the Atlanta stock on Instant Drosophila Medium (Colley, 1967).

Detection of Insemination

Parental females were recovered from each mass mating in set I on the eighth day after setting cultures and were etherized and dissected in Drosophila Ringer solution to expose the spermathecae and ventral receptacles. These organs were examined for the presence of sperm at 100x with a Microstar Series 10 microscope (American Optical Company). In crosses in which insemination was not detected on the eighth day after setting cultures, small mass matings consisting of five pairs were set and females were examined for insemination at 24 and 48 hr after setting

to determine whether initial insemination, followed by loss or degeneration of sperm, had occurred.

Examination for Mechanical Isolation

External terminalia of males and females of each strain were examined at 25x with a Cycloptic dissecting microscope (American Optical Company). External genitalia were measured with a Bausch and Lomb ocular micrometer calibrated with a Bausch and Lomb stage micrometer. Drawings were made of the external terminalia of typical males and females of each strain with a crow quill pen and India ink. The internal genitalia of females of each strain were dissected in Drosophila Ringer solution and were examined without cover glass at a magnification of 100x using the Microstar microscope. Ink drawings were prepared to illustrate differences among the strains with respect to internal genitalia of females.

Chromosomal Preparations

Third instar larvae (after darkening of anterior spiracles) were collected and salivary glands were dissected in Drosophila Ringer solution. The salivary glands were transferred to several drops of Strickberger's lacto-acetic orcein (Strickberger, 1962) on a glass slide. The glands were stained for 4-5 min, covered with a cover glass, and squashed by applying pressure with the thumb. The preparations were sealed with colorless nail polish and were examined with the Microstar microscope. Selected preparations were photographed at 1000x using a stationary Polaroid camera.

Statistical Methods

The 95% and 99% confidence limits for percentages of successful mating pairs per cross and percentages of females inseminated per cross were calculated with the aid of tables in Statistical Methods by Snedecor and Cochran (1967). The mean number of offspring per pair per cross and the standard error of the mean were calculated according to methods outlined in Biometry by Sokal and Rohlf (1969), which also was the source of methods for analysis of variance in the number of offspring per mass mating per cross.

CHAPTER III

RESULTS

Pair Matings

From the thirty pair matings per cross set in tube cultures, two sets of data were analyzed for each cross: (1) Number of successful pairs per cross and (2) mean number of offspring per successful pair. A successful pair is defined as a pair producing adult offspring. A significant difference is defined as nonoverlapping confidence limits at the 95% level. Interstrain crosses are designated by the letters "ar" followed first by the stock number of the female parent, then by the stock number of the male parent. For example, an interstrain cross between an ar 4 female and an ar 5 male is designated ar 45.

The percentages of successful pairs per cross and their 95% and 99% confidence limits are shown in Table 1. Figure 1 illustrates relationships between frequency of success of interstrain crosses and the stocks to which male and female parents belong. Interstrain crosses which were significantly different from both parental controls involved stock ar 11 and stock ar 12; in these crosses, either there were no successful pairs or there was only a single successful pair per cross. In crosses involving either ar 5 or ar 6 in combination with either ar 11 or ar 12 (ar 511, ar 115, ar 611, ar 116, ar 512, ar 125, ar 612, and ar 126), frequencies of success were significantly lower than frequencies for ar 5 and ar 6 parental controls at the 95% level

Table 1. Percentages of Pair Matings per Cross Producing Adult Offspring, with 95% and 99% Confidence Limits.

Stock Number of Female Parent		Stock Number of Male Parent 4	5	6	11	12	13
4	#/30 (%)	21 (70)	14 (47)	23 (77)	0 (0)	0 (0)	27 (90)
	C.L. 95	52-84	29-68	59-90	0-10	0-10	75-98
	C.L. 99	40-88	24-70	53-92	0-16	0-16	69-99
5	#/30 (%)	12 (40)	7 (23)	9 (30)	0 (0)	1 (3)	22 (73)
	C.L. 95	23-60	10-41	16-48	0-10	0-17	56-87
	C.L. 99	19-66	8-47	12-54	0-16	0-21	49-90
6	#/30 (%)	6 (20)	5 (17)	10 (33)	0 (0)	0 (0)	28 (93)
	C.L. 95	9-37	6-33	17-53	0-10	0-10	79-99
	C.L. 99	6-43	4-39	15-57	0-16	0-16	74-100
11	#/30 (%)	0 (0)	0 (0)	0 (0)	19 (67)	0 (0)	0 (0)
	C.L. 95	0-10	0-10	0-10	44-80	0-10	0-10
	C.L. 99	0-16	0-16	0-16	38-85	0-16	0-16
12	#/30 (%)	0 (0)	0 (0)	0 (0)	0 (0)	25 (83)	0 (0)
	C.L. 95	0-10	0-10	0-10	0-10	67-94	0-10
	C.L. 99	0-16	0-16	0-16	0-16	61-96	0-16
13	#/30 (%)	22 (73)	13 (43)	31* (88)	0 (0)	1 (3)**	25 (83)
	C.L. 95	56-87	24-64	74-97	0-10	0-17	67-94
	C.L. 99	49-90	20-68	70-98	0-16	0-21	61-96

*31 of 35 pairs.
**Offspring consisted of a single individual.

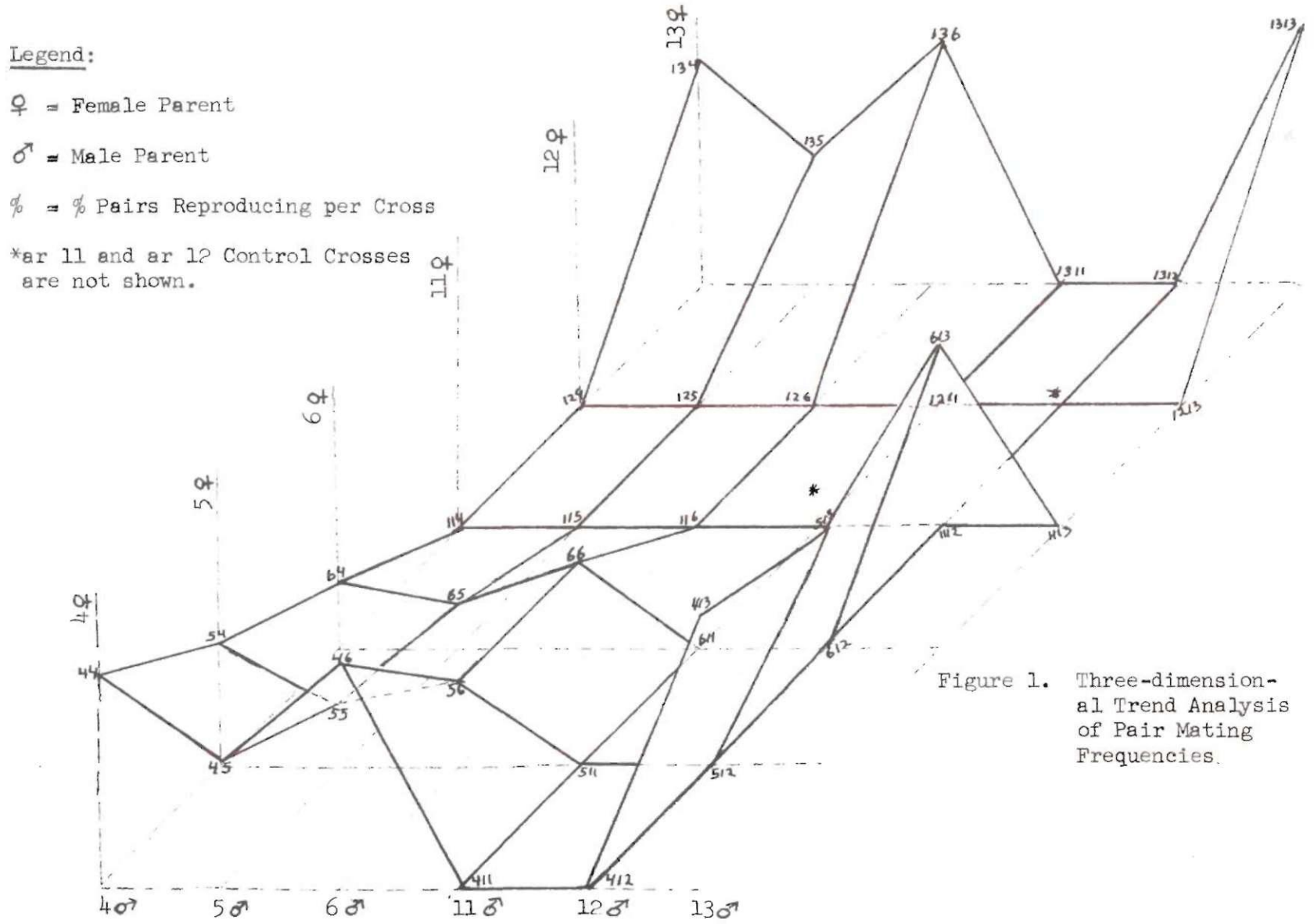

Figure 1. Three-dimensional Trend Analysis of Pair Mating Frequencies.

and were significantly lower than frequencies for ar 11 and ar 12 parental controls at the 99% level. These low frequencies might be attributed in part to the relatively low frequency of success in pair matings observed in parental controls ar 55 and ar 66 (Table 1). All other interstrain crosses involving either ar 11 or ar 12 were significantly different from both parental controls at the 99% level.

Interstrain crosses significantly different with respect to only one of the two parental controls were ar 136, ar 613, ar 135, ar 513, ar 46, and ar 64. In both ar 136 and the reciprocal cross, ar 613, frequencies of success were high, 88% and 93%, respectively. In both reciprocal crosses, frequencies were significantly different from the relatively low frequency of success in pair matings in the parental control cross ar 66 (33%). Both ar 135 and its reciprocal, ar 513, were significantly different from the control crosses (ar 55 and ar 1313) with respect to the female parent (Table 1). Ar 46 and its reciprocal, ar 64, were significantly different from controls with respect to the male parent. In addition, ar 46 and ar 64 were the only crosses in which there was a significant difference between reciprocals.

Mean numbers of offspring per successful pair for each cross are shown in Figure 2. The amount of variance for each cross is associated with the size of N. Only two crosses were found to be significantly different from parental controls with respect to the mean number of offspring per successful pair. In ar 46, the mean number of offspring was significantly higher than in the parental control ar 44 but was not significantly higher than in the parental control ar 66. The mean

Legend:

Vertical Axis: Mean number of offspring per successful pair.
Horizontal Axis: Identification of interstrain and control crosses.
Number in parentheses beneath each cross represented on horizontal axis is the number of successful pairs per cross.

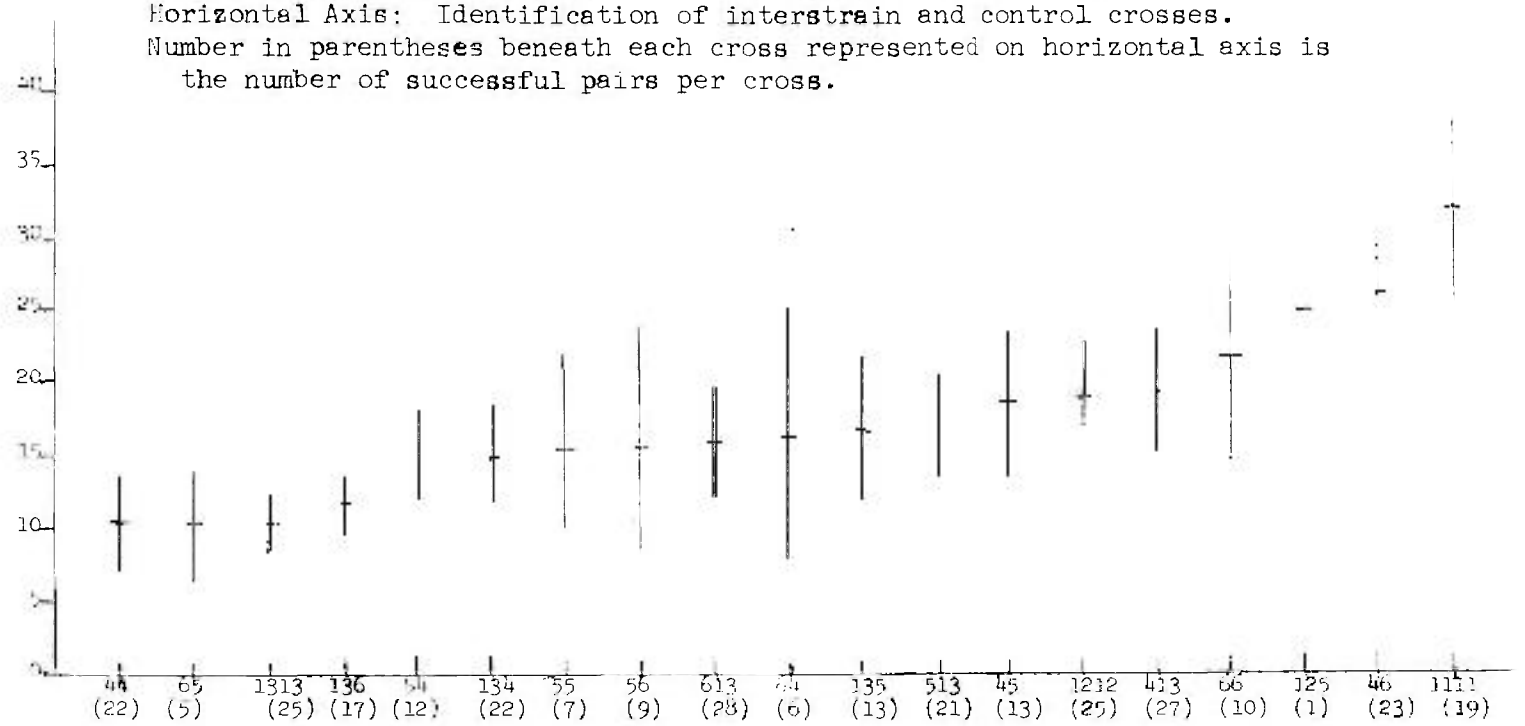

Figure 2. Mean Numbers of Offspring per Successful Pair per Cross ± Two Standard Errors of the Mean.

number of offspring in the reciprocal cross, ar 64, was intermediate between means for parental controls. In ar 413, the mean number of offspring was significantly higher than in either ar 1313 or ar 44. The mean number of offspring in the reciprocal cross, ar 134, was also higher than in either control cross, but the difference was not significant at the 95% level.

F_1 x F_1 crosses were set using the offspring of each pair mating. The F_1 generation of all crosses was fertile, and no differences were observed among F_1 hybrid stocks in frequencies of successful F_1 x F_1 crosses.

Mass Matings

Two sets of mass mating experiments, in which each mass mating consisted of 30 pairs per bottle, were performed for each cross. In only two cases did a cross which was unsuccessful in pair matings succeed in mass matings. These crosses were ar 411 in set I and ar 116 in set II. The number of offspring per bottle was counted and an analysis of variance was run to determine whether there were any significant differences among fertile crosses with respect to numbers of offspring produced. The variance between successful crosses was not significantly different from variance within crosses at the 0.05 level (Appendix).

Insemination of Females

Females were removed from mass mating bottle cultures for dissection on the eighth day after setting, and spermathecae and ventral

receptacles were examined microscopically for the presence of sperm. Percentages of females inseminated per cross and their 95% and 99% confidence limits are shown in Table 2. Females from stock ar 11 and stock ar 12 were inseminated only by males from the same stocks. Of ar 6 females examined, 53% were inseminated when crossed to ar 4 males; 48% of ar 5 females and 12% of ar 13 females were inseminated when crossed to ar 11 males. These crosses were the only three in which percentages of females inseminated were significantly different from 100%. All crosses involving ar 11 females and ar 12 females were reset in small mass matings consisting of five pairs per bottle. Females were removed and dissected at intervals of 24 hr and 48 hr, and spermathecae and ventral receptacles were examined. In no instance was insemination detected.

When crossed to ar 12 males, females of all stocks except ar 12 exhibited distinctly discernible decreases in numbers of spermatozoa in spermathecae and ventral receptacles, as compared to numbers of sperm observed in matings with males of other stocks.

Comparison of External Terminalia

Examination of the external terminalia of females and males of each parental stock revealed strain-specific morphologies. The external terminalia of each stock are illustrated in Figures 3-5. Differences in size and orientation of external genitalia which might be significant with respect to mechanical isolation, including differences in length of penis, length of vaginal plate, and distance and angle between these structures and the anal plate, were defined as differences between

Table 2. Percentages of Females Inseminated in Each Cross, with 95% and 99% Confidence Limits.

Stock Number of Female Parent		Stock Number of Male Parent 4	5	6	11	12	13
4	%	100	100	100	94	100	100
	C.L. 95	86-100	87-100	87-100	76-100	85-100	87-100
	C.L. 99	80-100	84-100	83-100	70-100	79-100	83-100
5	%	100	96	100	48	63	95
	C.L. 95	80-100	83-100	81-100	28-64	43-74	80-100
	C.L. 99	74-100	77-100	74-100	23-67	36-76	74-100
6	%	53	82	100	100	93	100
	C.L. 95	38-64	58-90	82-100	87-100	75-94	87-100
	C.L. 99	30-67	45-92	75-100	83-100	67-94	83-100
11	%	0	0	0	100	0	0
	C.L. 95	0-21	0-21	0-11	88-100	0-27	0-16
	C.L. 99	0-30	0-30	0-15	87-100	0-31	0-22
12	%	0	0	0	0	100	0
	C.L. 95	0-12	0-12	0-11	0-14	88-100	0-11
	C.L. 99	0-17	0-17	0-15	0-20	83-100	0-16
13	%	100	100	100	42	80	100
	C.L. 95	88-100	80-100	89-100	18-61	68-80	89-100
	C.L. 99	83-100	74-100	85-100	15-65	63-80	85-100

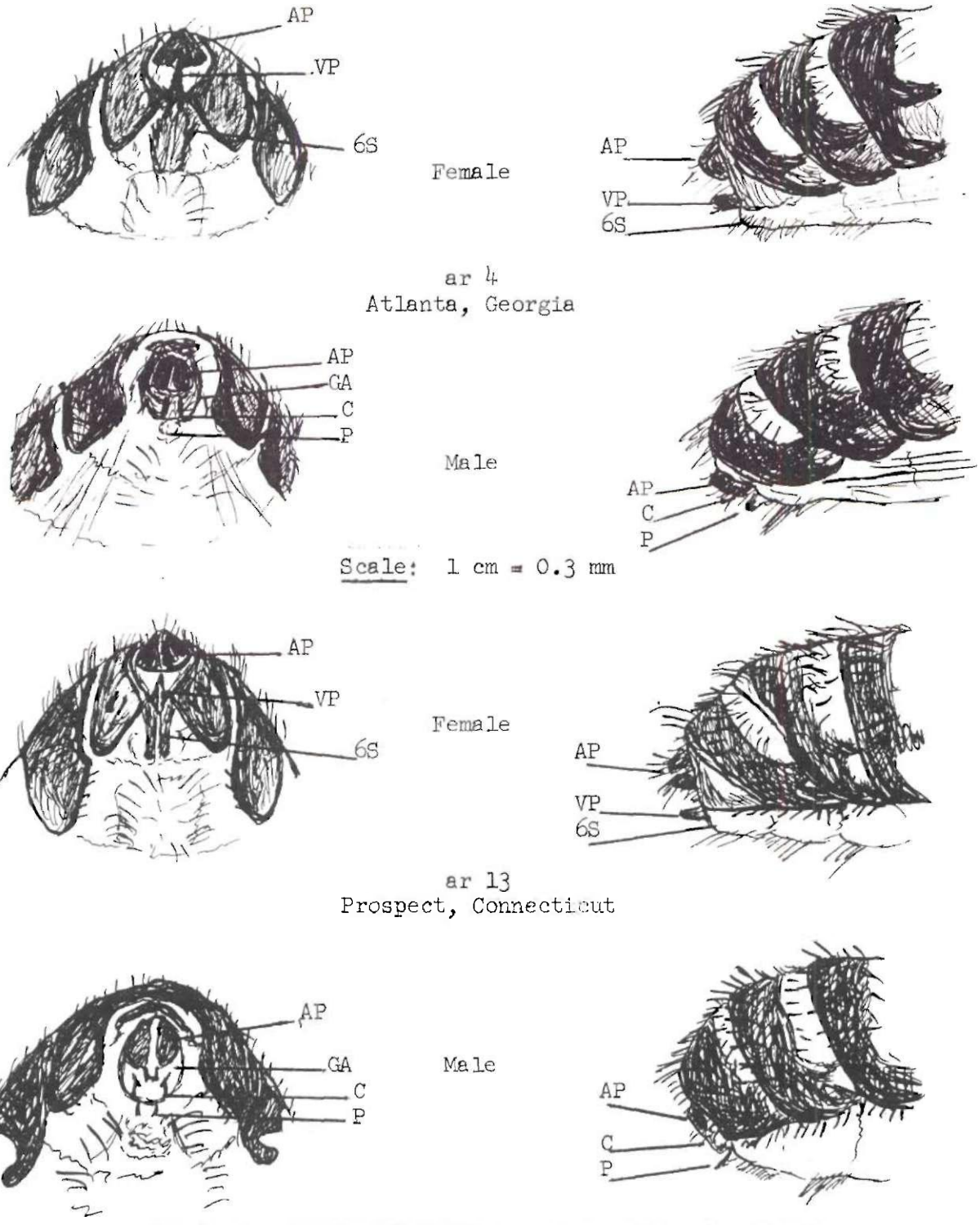

Figure 3. Ventral and Lateral Views of Female and Male External Terminalia of ar 4 (Atlanta, Georgia) and ar 13 (Prospect, Connecticut). Legend: AP = anal plate; VP = vaginal plate; 6S = sixth sternite; GA = genital arch; C = claspers; P = penis.

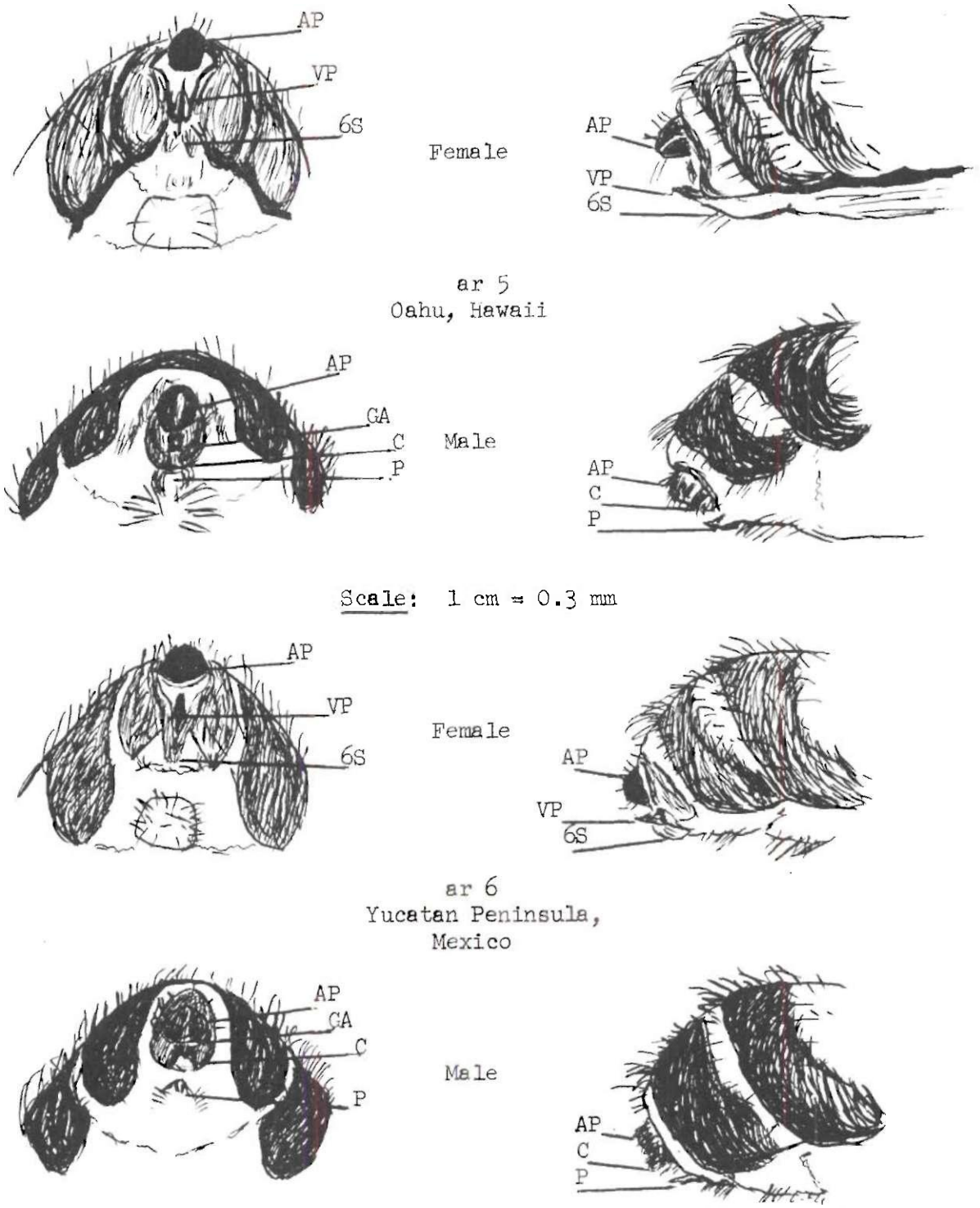

Figure 4. Ventral and Lateral Views of Female and Male External Terminalia of ar 5 (Oahu, Hawaii) and ar 6 (Yucatan Peninsula, Mexico). Legend: AP = anal plate; VP = vaginal plate; 6S = sixth sternite; GA = genital arch; C = claspers; P = penis.

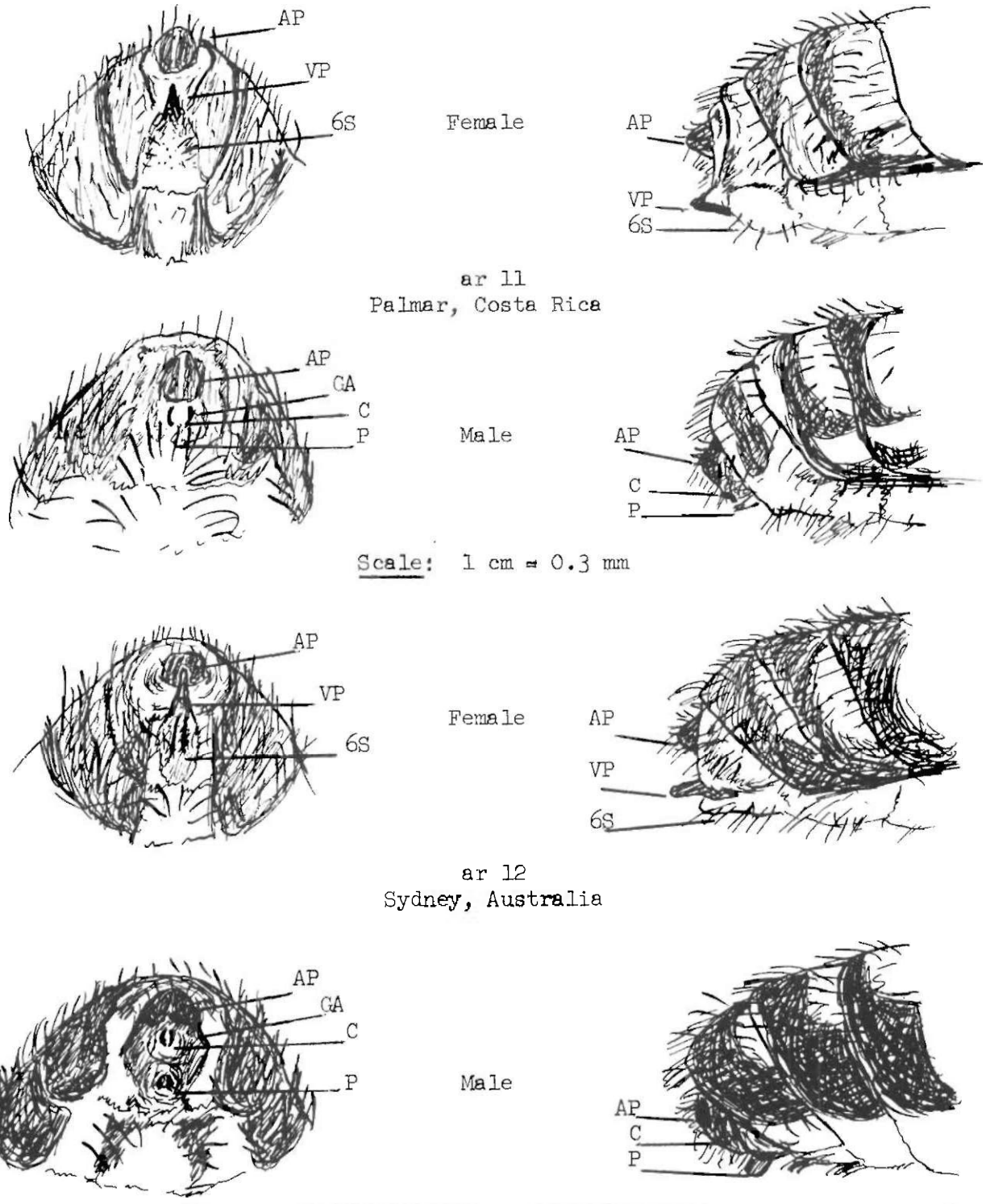

Figure 5. Ventral and Lateral Views of Female and Male External Terminalia of ar 11 (Palmar, Costa Rica) and ar 12 (Sydney, Australia). Legend: AP = anal plate; VP = vaginal plate; 6S = sixth sternite; CA = genital arch; C = claspers; P = penis.

strains observed to be greater than differences within strains. The examination revealed no such differences which would indicate mechanical isolation resulting from differences in external genitalia among these strains.

Comparison of Female Internal Genitalia

Examination of internal genitalia of dissected females of each strain revealed no visually detectable differences except with respect to spermathecae and ventral receptacles. Spermathecae of ar 4, ar 5, ar 6, and ar 13 females exhibited a common structure, illustrated in Figure 6a. Ar 12 and ar 11 females exhibited marked differences from other stocks with respect to spermathecal structure, as depicted in Figures 6b and 6c, respectively. The "coiled spring" ventral receptacle is found in all strains except ar 11; however, differences in numbers of coils were observed among strains. Ar 4 and ar 5 have approximately 30 coils; ar 6 and ar 13 have approximately 60 coils; and ar 12 has approximately 150 coils (Fig. 7a). The ventral receptacle of ar 11 is illustrated in Figure 7b.

Developmental Arrest

Parental flies from set II of the mass matings were used in studies designed to detect the occurrence of developmental arrest. From each interstrain cross which had not previously produced adult offspring and from each control cross, at least five pairs were transferred to chambers which permitted oviposition on petri dishes containing Instant Drosophila Medium. Plate cultures were examined at 24 hr

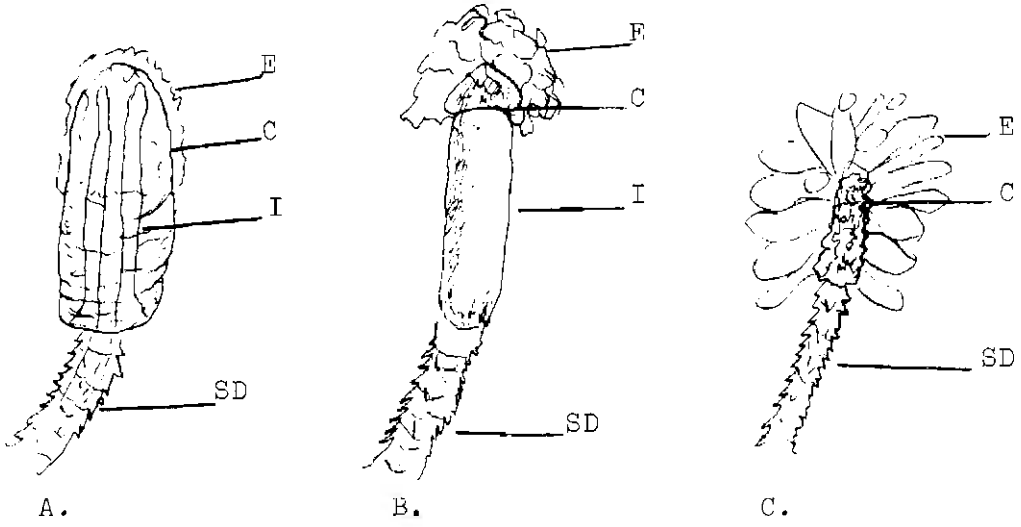

Figure 6. Spermathecae of D. repleta Strains. A. ar 4, ar 5, ar 6, and ar 13. B. ar 12. C. ar 11.
Legend: C = sclerotized capsule; SD = spermathecal ducts; E = envelope; I = introvert. (Scale: 1 cm = 0.1 mm)

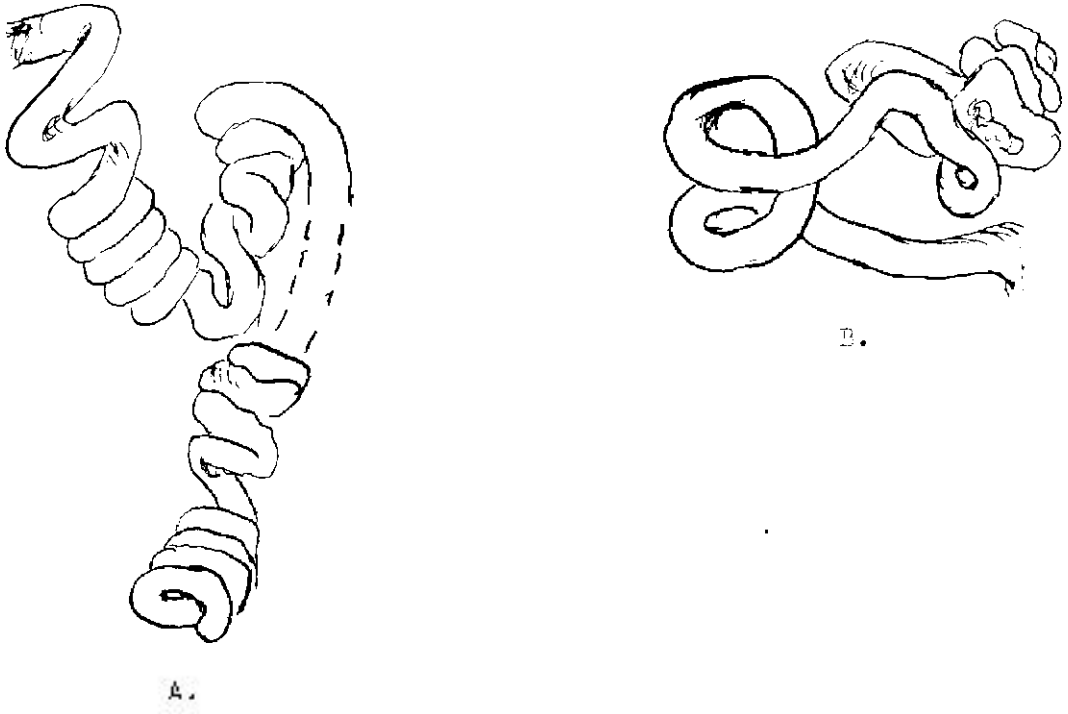

Figure 7. Ventral Receptacle Types in D. repleta Strains. A. ar 4, ar 5, ar 6, ar 12, ar 13. B. ar 11.

intervals for evidence of developmental arrest. In all crosses, females oviposited, and all control crosses produced adult offspring. Interstrain crosses producing adult offspring were ar 512, ar 125, ar 612, ar 126, ar 1311, and ar 1312. In those interstrain crosses which did not yield adult offspring, only unhatched eggs were observed.

Chromosomal Studies

Examination of larval salivary gland chromosomes of each stock revealed neither inversions nor any other visible rearrangement. In general, examination of salivary gland chromosomes of offspring of interstrain crosses also revealed no configurations indicating aberrations; however, in several larvae from cross ar 513, nonpairing of chromosome ends was observed. These unpaired ends give the chromosome the appearance of the letter "Y," as shown in Figure 8.

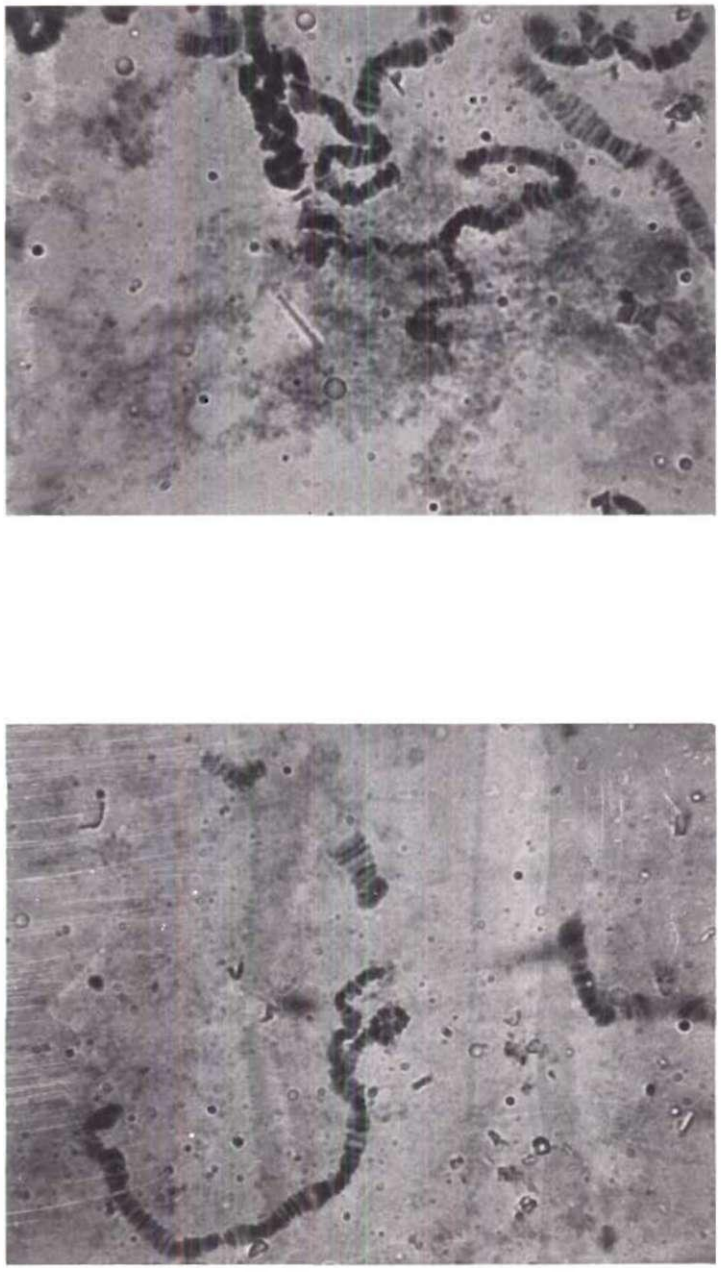

Figure 8. Non-pairing of Ends of Polytene X Chromosomes in a Female ar 513 Hybrid Larva (X450).

CHAPTER IV

DISCUSSION

Figure 1 illustrates the trends observed in analysis of data from pair matings. The statistically significant failure of ar 11 and ar 12 to produce offspring in any interstrain cross is strikingly visible. In addition, other trends emerge which are less readily detected in confidence limit analysis (Table 1). All successful interstrain crosses involving ar 13 exhibit a high frequency of success in pair matings, as shown by peaks for ar 13 on both the male axis and the female axis (Fig. 1). Interstrain crosses involving either ar 5 or ar 6 tend to exhibit low frequencies of success in pair matings. For example, the frequency of success in ar 135 is high for a cross involving ar 5 but is very low in comparison to other interstrain crosses involving ar 13. The parental control crosses ar 55 and ar 66 also exhibit comparatively low frequencies of successful pair matings per cross. These low frequencies might be attributed to a temperature of incubation in laboratory culture (23 ± 1 °C) lower than the optima for these strains. Both ar 5 and ar 6 originally were collected in regions with warm climates, and the temperature of incubation may be far enough below their optimum temperatures to interfere with reproduction; such effects of temperature on reproduction have been described for other species by Spencer (1950). Alternatively, there might exist in these

strains a genetically determined behavioral disaffinity to mating in pair matings; lack of success in pair matings was reported to be characteristic of the species by Wharton (1942).

The mean number of offspring per successful pair is indicated for each cross in Figure 2. The size of the standard error of the mean did not appear to be related either to the particular strain or to the hybrid condition. In most cases, standard error was inversely proportional to the size of N.

Inspection of mean numbers of offspring per successful pair (Fig. 2) in conjunction with frequencies of successful matings (Table 1) revealed that both parental cross ar 44 and parental cross ar 1313 have high frequencies of success in pair matings and low mean numbers of offspring per pair. Ar 1111 has the highest mean number of offspring per pair (32) and a moderately high frequency of successful pairs (67%). Ar 1212 has a moderately high mean number of offspring (22) and a high frequency of success in pair matings (83%). These frequencies are consistent with observations during routine laboratory culture of stocks. Both ar 11 and ar 12 produce large numbers of offspring and the life cycle is completed within 12-13 days (personal communication, A. M. Colley, M. K. Jocoy). In laboratory stock cultures, ar 5 and ar 6 supply relatively large numbers of offspring; however, the cultures require at least 48 hr longer to complete the life cycle, again possibly reflecting temperature effects (Spencer, 1950).

Ar 413 and ar 46 were the only two interstrain crosses in which mean number of offspring per pair was greater than in either parental

control cross. The mean number of offspring per pair for the reciprocal cross, ar 134, was also greater than for either parental control, although the difference was not significant at the 95% confidence level. These results are interpreted as indicating hybrid vigor in progeny of these interstrain crosses.

Mass matings were set using 30 pairs per bottle. For crosses which had been highly successful in pair matings, this number was excessive and led to crowding during early development, which resulted in the production of fewer adult offspring per female than were obtained from pair matings. In many instances, crosses with low mean numbers of offspring in pair matings yielded a mean number of offspring per 30 females in mass matings higher than mass mating yields for crosses which had been highly prolific in pair matings. For example, ar 1212 ($\bar{X}$ = 22 offspring per pair) produced only 17-22 adult offspring per 30 females in mass matings, while ar 65 ($\bar{X}$ = 10 offspring per pair) produced 45-190 adult offspring per 30 females in mass matings. In general, by reducing the number of offspring in successful crosses, overcrowding served to minimize any differences in number of offspring per cross which might have been observed among crosses. The number of repetitions of mass mating experiments was small and the error correspondingly large, so that analysis of variance revealed no significant difference in variances in numbers of offspring per 30 females between and within mass mating crosses at the 0.05 level (Appendix).

Frequencies of insemination of females were not significantly different from 100% at the 95% confidence level, except for interstrain crosses involving ar 11 females and ar 12 females and crosses ar 46,

ar 511, and ar 1311 (Table 2). These observations indicate that pre-copulatory isolating mechanisms were not operating in the majority of crosses, in contrast to observations of Wharton (1942) for Texas, Ankara, Guatemala, and New Haven strains of D. repleta.

The sexual isolation observed in cross ar 46 probably accounts for the significant difference in frequency of success in pair matings between this cross and its reciprocal, ar 64, since the mean number of offspring per pair (Fig. 2) does not indicate hybrid mortality. Insemination was not observed in any interstrain cross involving either ar 11 females or ar 12 females; thus, their total failure to produce offspring in interstrain pair matings can probably be attributed to pre-copulatory isolating mechanisms. Both ar 12 males and ar 11 males also are unsuccessful in interstrain crosses. The consistently small numbers of sperm observed in the female genital tract in interstrain crosses in which females were inseminated by ar 12 males implicates gametic mortality as a factor in crosses with ar 12 males.

Examination of female internal genitalia revealed structural differences among strains only with respect to spermathecae and ventral receptacles (Figs. 6 and 7). The structure of spermathecae has undergone many modifications during the evolution of Drosophila species (Patterson and Stone, 1952; Throckmorton, 1962). In construction of schemes of phylogeny in the genus Drosophila, morphological variation in spermathecae and ventral receptacles of different species has been analyzed in conjunction with variation in other reproductive structures (Throckmorton, 1962).

The spermathecae found in ar 4, ar 5, ar 6, and ar 13 (Fig. 6a) are morphologically identical to descriptions of Patterson (1943) and Throckmorton (1962) for D. repleta. The spermathecae of ar 12 (Fig. 6b) are similar in shape to descriptions for D. neohydei (Patterson, 1943; Throckmorton, 1962). Small, rough, untelescoped spermathecae observed in ar 11 females (Fig. 6c) are considered to be the primitive morphological type for the genus (Throckmorton, 1962). The "coiled spring" ventral receptacle found in ar 4, ar 5, ar 6, ar 12, and ar 13 (Fig. 7a) is the form classically described for the species (Patterson, 1943; Throckmorton, 1962). The short, folded ventral receptacle of ar 11 (Fig. 7b) is considered more primitive than the "coiled spring" structure (Throckmorton, 1962).

Genetic isolation among strains might be attributed, at least in part, to such morphological differences. Spermathecae have been found to be necessary for the survival of sperm in the female genital tract (Anderson, 1945). Infertility of females of the lozenge mutant of D. melanogaster has been demonstrated to result from a partial or total absence of spermathecae. Spermatozoa present in the ventral receptacle become non-motile one to several days after copulation in females without spermathecae. Anderson suggested that spermathecae are the site of synthesis of a substance necessary for continued viability and motility of sperm. The small numbers of sperm found in females inseminated by ar 12 males in the present study might reflect a biochemical incompatibility between sperm and spermathecae of different strains.

The examination of external terminalia revealed no differences

in size of external genitalia sufficiently great to indicate the operation of mechanical isolation among these strains. Nevertheless, the terminalia of each strain exhibit characteristic, strain-specific morphologies in each sex (Figs. 3-5). Ar 11 has a distinctly lighter body color and brighter red eye color than do other strains. The other strains are less readily distinguishable morphologically; however, on closer inspection they exhibit structural differences. For example, the light spot on the second-to-last tergite of ar 4 is characteristic of the strain (Fig. 3).

Other research in progress using these stocks indicates the occurrence of quantitative differences among strains in adult pteridine patterns (personal communication, A. M. Colley) and strain-specific developmental timing of appearance and disappearance of a number of compounds in pteridine metabolism, correlated with strain-specific morphological developmental cycles with varying intervals spent in each stage in the life cycle (personal communication, M. K. Jocoy). Ar 11 and ar 12 exhibit the greatest differences from other strains with respect to developmental timing of both morphological and biosynthetic changes.

In experiments in the present study investigating time of developmental arrest, all crosses set in petri dish cultures yielded either adult offspring or unhatched eggs. All parental control crosses produced adult offspring. In addition, interstrain crosses ar 512, ar 125, ar 612, ar 126, ar 1311, and ar 1312 produced adult offspring. Since Drosophila are known to lay unfertilized eggs (Sonnenblick, 1950), the

laying of unhatched eggs by females of all unsuccessful crosses, even those in which females were inseminated, provided no conclusive information as to whether or not fertilization had occurred. In any event, the occurrence of developmental arrest beyond the egg stage was not observed.

The presence of adult offspring in petri dish cultures of six crosses not previously observed to reproduce is probably due to differences in environment provided by different methods of culture. Eggs and first instar larvae co-exist in a delicate balance with yeast in culture. Normally, the eggs hatch after 24-48 hours in these strains (Colley, 1967; personal communication, M. K. Jocoy), and larvae begin to control the yeast population. However, if eggs are not laid or fail to hatch before the yeast multiplies excessively, a coating of yeast is formed over the surface of the medium, covering eggs and preventing normal respiratory exchange through the egg surface. The results are similar if the number of larvae is very small; the yeast takes over the culture, and eggs and early larval instars are "suffocated." Filter paper discs covering the surface of the medium in petri dish cultures separated and protected eggs and larvae from the yeast "coating," while permitting larvae to feed through the paper. In the cases in which crosses produced no adult offspring in pair mating tubes or bottle cultures but did produce adults in plate culture, it seems probable that these crosses in fact produced small numbers of larvae in tubes and bottles but that the larvae died before reaching the third instar, at which time their size would have rendered them detect-

able even if present in small numbers; thus, it is possible that isolation is not complete between any of these strains.

Examination of lacto-acetic orcein-stained salivary gland chromosome smears revealed no inversions or other rearrangement configurations in parental stocks. The chromosomes exhibited a tendency to break, but several interpretable preparations were obtained for each stock. In interstrain hybrids examined (ar 46, ar 64, ar 65, ar 512, ar 135, ar 513, and ar 45), there were no positive indications of rearrangements, although in ar 513 preparations, Y-shaped configurations were observed (Fig. 8). These configurations were identified as the X chromosomes of female larvae with ends unpaired. Banding patterns of the unpaired ends of the homologues appeared to be identical. A similar phenomenon has been observed in interspecific hybrids (Painter and Stone, 1935) and is reported to reflect incompatibility of homologues, which might be due to structural differences in histone I fractions of different species or strains or to cytologically indetectable differences in the genetic material itself.

The results of this study indicate that no strain is completely isolated genetically from all of the other strains and that sexual isolation, which Wharton (1942) considered to be the major mechanism of genetic isolation in D. repleta, is at least one of the isolating mechanisms operating to prevent or reduce the frequency of interbreeding among these strains. In contrast, sexual isolation among strains has not been detected in D. pseudoobscura. In a study of strains from widely different localities (British Columbia, Canada;

Colorado; California; Texas; and Sonora, Mexico), Anderson and Ehrman (1969) found no deviation from random mating. In another study, which included a strain from Bogata, Columbia, Prakash (1972) reported evidence of partial reproductive isolation in D. pseudoobscura. In crosses of strains from California, Texas, Colorado, and Guatemala with each other and with the Bogata strain, random mating was observed; however, Bogata females crossed with males of any other strain yielded sterile F_1 males. The normal range of D. pseudoobscura does not extend south of Guatemala (Prakash, 1972), so that, for the Bogata strain, gene exchange with other strains through geographically intermediate populations is precluded. The author concluded that complete geographical isolation, in conjunction with founder effects, leads to reproductive isolation.

Comparing the results of Prakash (1972) to those of the present study reveals some interesting parallels. Although sexual isolation, rather than hybrid sterility, is the reproductive isolating mechanism in question, the factors leading to establishment of isolating mechanisms appear to have been similar. The North American and Mexican strains of D. repleta interbred freely. The inclusion of the Hawaiian stock, ar 5, in this group suggests its relatively recent introduction from the mainland. The two strains exhibiting marked reproductive isolation were collected in geographically isolated localities in which gene exchange with populations from the other regions is virtually precluded. The collection site of the Australian stock, ar 12, not only is the most distant from collection sites for other stocks, but also is isolated

from these regions by the Pacific Ocean. The Costa Rican stock, ar 11, was collected from a region which is less distant from the North American mainland but which is isolated by the Pacific Ocean and the Cordeрilla mountain range.

Thus, while an absolute correlation between distance of geographical separation and reproductive isolation among stocks was not observed, reproductive isolation was found to be correlated with the existence of geographical barriers (e.g., the Pacific Ocean) which one would expect to have prevented gene exchange through intermediate populations. In view of results of this study, it seems reasonable to conclude that geographical isolation among these strains has been accompanied by genetic isolation potentially leading to speciation and to suggest a reevaluation of the status of Australian and Costa Rican strains as incipient species.

CHAPTER V

CONCLUSIONS

Results of this study suggest that geographical isolation among these strains has been accompanied by genetic isolation. Strain-specific morphologies indicate genetic differences among stocks. Ar 4 (Atlanta, Georgia), ar 5 (Oahu, Hawaii), ar 6 (Yucatan Peninsula, Mexico), and ar 13 (Prospect, Connecticut) appear to constitute a group, members of which exhibit relatively few morphological and developmental differences. Ar 11 (Costa Rica) and ar 12 (Australia) represent extreme types having many characteristics different from those of other strains. Not only do ar 11 and ar 12 fail to interbreed in pair matings with other stocks, but they exhibit marked differences in structure of internal and external genitalia and have temporal differences in developmental cycles. Sexual isolating mechanisms operating between ar 11 and ar 12 and the other strains were the only genetic isolating mechanisms conclusively demonstrated to be operating among these strains.

While an absolute correlation between distance of geographical separation and reproductive isolation among stocks was not observed, reproductive isolation was found to be correlated with the existence of geographical barriers (e.g., the Pacific Ocean) which one would expect to have prevented gene exchange through intermediate populations. In view of results of this study, it seems reasonable to conclude that

geographical isolation of Australian and Costa Rican strains has been accompanied by genetic isolation potentially leading to speciation and to suggest a reevaluation of the status of these two strains as incipient species.

CHAPTER VI

RECOMMENDATIONS

Several lines of experimental work which would elucidate questions raised by results of this study are recommended:

1. Determination of frequencies of insemination in interstrain pair matings.

2. Cytological examination of eggs to determine whether fertilization has occurred.

3. Comparison of frequencies of success and mean numbers of offspring in pair matings at several different temperatures.

4. More detailed morphological comparison between strains and morphological characterization of interstrain hybrids.

5. Increased number of repetitions of mass matings, using fewer pairs per bottle, or conduction of mass matings in large population cages.

6. More detailed analysis of results of $F_1 \times F_1$ crosses, including examination of F_2 and subsequent generations for hybrid breakdown.

7. More detailed chromosome studies of F_1 interstrain hybrids.

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APPENDIX

Table 1. Numbers of Offspring and Results of Analysis of Variance in Successful Interstrain and Control Mass Matings

Cross	Number of Offspring			
	Mass Mating I	Mass Mating II	Total (X)	$(X)^2$
ar 44	64	100	164	26896
ar 45	135	120	255	65025
ar 46	120	245	365	133225
ar 411	36	0	36	1296
ar 413	24	110	134	17956
ar 55	55	17	72	5184
ar 54	12	38	50	2500
ar 56	91	53	144	20736
ar 513	36	60	96	9216
ar 66	65	100	165	27225
ar 64	47	85	132	17424
ar 65	190	45	235	55225
ar 613	55	40	95	9025
ar 1111	164	140	304	92416
ar 116	0	90	90	8100
ar 1212	17	22	39	1521
ar 1313	42	96	138	19044
ar 134	71	35	106	11236
ar 135	39	55	94	8836
ar 136	79	52	131	17161

Table 1, continued, Calculations:

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(1) $\sum X = 2,845$

(2) $\sum X^2 = 519,547$

(3) $(\sum X)^2 = 8,094,025$

(4) Correction Factor:

$$C = \frac{(\sum X)^2}{N \times Z} = \frac{8,094,025}{20 \times 2} = 202,350.6,$$ where N = number of groups and Z = number of repetitions.

(5) Corrected Between-Group Variance:

$$V_B = \frac{\frac{\sum X^2}{Z} - C}{N-1} = \frac{\frac{519,547}{2} - 203250.6}{19} = \frac{259,773.5 - 202,350.6}{19}$$

$$= \frac{57,422.9}{19} = 3025.6$$

(6) Within-Group Variance:

$$V_W = \frac{\frac{\sum X^2}{Z}}{(N)(Y)(Z-1)} = \frac{\frac{519,547}{2}}{(20)(1)(2-1)} = \frac{259,773.5}{20} = 12,988.7,$$

where Y = number of treatments; here, Y = 1.

(7) $f = \frac{V_B}{V_W} = \frac{3025.6}{12,988.7} = 0.232$

$f_{0.05} = 4.14$ (Sokal and Rohlf, 1969).

$f < f_{0.05}$; thus, variances in numbers of offspring per 30 females in mass matings between and within groups (crosses) are not significantly different at the 0.05 level.